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Detection of Antioxidative Activity of Plant Extracts at the DNA-Modified Screen-Printed Electrode

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Abstract: A simple procedure for the voltammetric detection of antioxidative activity of plant extracts based on the protection from DNA damage at the electrode surface is reported. A disposable electrochemical DNA biosensor fabricated as a carbon-based screen-printed electrode modified by a surface layer of the calf thymus double stranded (ds) DNA was used as a working electrode in combination with a silver/silver chloride reference electrode and a separate platinum auxiliary electrode. The $[Co(phen)_3]^{3+}$ ion served as the dsDNA redox marker and the $[Fe(EDTA)]^-$ complex with hydrogen peroxide under the electrochemical reduction of the iron atom were used as the DNA cleavage mixture. A remarkable antioxidative activity of phenolic antioxidants such as rosmarinic and caffeic acids as standards and the extracts of lemon balm, oregano, thyme and agrimony was found which is quite in agreement with an antiradical activity determined spectrophotometrically using 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical.

Keywords: DNA biosensor, Screen-printed electrode, Damage to DNA, Antioxidants, Plant extracts

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1. Introduction

Antioxidants are substances which at low concentrations compared to that of an oxidazable substrate markedly delay or prevent the oxidation of the substrate [1]. They are used in the food industry to delay the oxidation process [2, 3]. The most active dietary antioxidants belong to the family of phenolic and polyphenolic compounds. Phenolic acids are widely distributed in the plant kingdom. An antioxidative activity of a particular compound, a mixture of compounds, or a natural source containing such compounds, is generally related to its (their) ability to scavenge free radicals, decompose them, or to quench singlet oxygen or possibly act as metal chelators or synergists with other components present [4]. The antioxidative activity is usually estimated by optical methods, e.g., photochemiluminometry [5] and absorption spectrophotometry with a stable free radical 2,2'-diphenyl-1-picrylhydrazyl (DPPH) [6, 7].

Many natural and synthetic antioxidants are known to exhibit an effective protection against the DNA damage by reactive oxygen and other species [8]. These species oxidize the DNA bases and deoxyribose and lead to a release of the bases and an interruption of the phosphodiester bonds. Therefore, the utilization of a DNA-based sensor for the evaluation of antioxidants is of interest. A ferrous complex of EDTA is often used to generate hydroxyl radicals in reaction with hydrogen peroxide [9]. In this Fenton-type reaction, ascorbic acid is typically added to reduce iron(III) and maintain a oxidation/reduction cycle of the iron atom. In such case it is rather difficult to differentiate between antiradical and metal-reduction properties of ascorbic acid as well as phytochemicals. Alternatively, an electrode potential can be used for the reduction of the transition metal which allows to omit the presence of ascorbic acid in the cleavage mixture. A potential-modulated cleavage of the surface-confined DNA was already reported [10] and we use this approach of iron electroreduction advantageously in this work.

Electrochemical DNA biosensors with an immobilized layer of the DNA discriminator are already known as simple and sensitive bioanalytical devices for the detection of DNA damage. The mercury electrode exhibits a high sensitivity to changes in the DNA structure [11]. Solid-phase electrodes in a combination with sensitive detection techniques become of interest as modern nucleic acids probes [12-15]. Disposable DNA sensors based on screen-printed electrodes (SPE) are also developed [16-18]. Recently, we have used the DNA-modified screen printed electrode for the evaluation of an antioxidative activity of yeast polysaccharides [19].

In this paper a scheme for the voltammetric detection of antioxidative activity of phenolic acids as phytochemicals at the DNA-modified carbon-based screen-printed electrode is presented. This scheme is based on the known quantification of the original double stranded (ds) DNA using the $[Co(phen)_3]^{3+}$ complex ion as a dsDNA electrochemical label. The amount of $[Co(phen)_3]^{3+}$ bound to the DNA layer decreases with a decrease in the portion of original dsDNA in the course of cleavage reaction [20, 21]. Two standards of phenolic acids as well as four aqueous plant extracts were tested as the antioxidants.

2. Experimental

2.1 Apparatus and Reagents

A computerized voltammetric analyzer ECA pol, model 110 (Istran, Bratislava, Slovakia) fitted with a screen-printed bielectrode system (FACH, Prešov, Slovakia) including a working electrode (25 mm^2 surface area) together with a silver/silver chloride reference electrode and a separate platinum counter electrode was used for the differential pulse voltammetric (DPV) measurements. The voltammetric measurements were carried out in a 10 ml glass one-compartment voltammetric cell at room temperature (22 °C).

UV-visible spectrophotometer UV-1601 (Shimadzu, Japan) was used for optical measurements of the radical DPPH. HPLC analysis was performed with a HP 1100 system (Hewlett-Packard, Waldbronn, Germany) consisting of a pump with degasser, a diode-array detector (DAD) and a HP ChemStation as reported previously [22]. Chromatographic column Symmetry C-18 (150×3.9 mm; 5 μm) and precolumn (20×3.9 mm) packed with the same sorbent (Watrex Corp., USA) were used for HPLC separation with a gradient mobile phase MeOH-water (pH = 2.5 adjusted with formic acid): 0-2 min (15:85) and 2-25 min (15:85 to 45:55) were used for the chromatographic separation. The flow rate was 0.4 ml/min and injected volume 20 ml. All analyses were carried out at ambient temperature. UV spectra were recorded in the range of 200-400 nm. Chromatograms were acquired at 260, 280 and 330 nm.

Calf thymus dsDNA was obtained from Merck (1.24013.0100) and used as received. Its stock solution (5 mg/ml) was prepared in 10 mM Tris-HCl and 1 mM EDTA solution of pH 8.0 and stored at –4 °C. The complex compound and $[Co(phen)_3](ClO_4)_3$ was synthesized in our laboratory according to Ref. [23] and checked by chemical analysis. Rosmarinic acid was from Q-Chem (Bratislava), caffeic acid from Sigma and their stock solutions were prepared in methanol. The 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH) was from Aldrich and for measurements its methanolic stock solution of 0.0251 g/100 ml was diluted 1+9 with methanol. Individual plant extracts were obtained by the extraction of 2.0 g of the corresponding dry plant powder with 100 ml of water at 70 °C for 1 hour. Deionized and double distilled water was used throughout.

2.2 Preparation of the modified electrode

The screen-printed working electrode (SPE) was prepared commercially (FACH, Prešov, Slovakia) using a common carbon ink (Electra Polymers and Chemicals, Tonbridge, UK). A surface of the bare strip without any electrochemical preconditioning was modified in laboratory by covering with 5 μl of the DNA stock solution and leaving the electrode to dry overnight.

2.3 Procedure

The DNA-modified screen-printed electrode (DNA/SPE) was immersed to 10 mM phosphate buffer pH 7.0 for 15 min and rinsed with water. Then, the $[Co(phen)_3]^{3+}$ marker was accumulated from its 5×10^{-7} M solution in 10 mM phosphate buffer under stirring for 120 s at an open circuit. The DP voltammogram was recorded immediately (without a medium exchange) from +0.4 to –0.5 V at the

pulse amplitude of 100 mV and the scan rate of 25 mV/s. With the software used, the current was measured with 2 mV scan step at this scan rate.

The marker DPV peak current (I_0) at –0.130 V was evaluated against a base-line using the standard software and corrected to the signal at a bare electrode surface by the subtraction of the mean marker DPV peak current measured at the SPE (n = 10) under the same conditions. Then, the DNA/SPE was regenerated by a removal of the accumulated $[Co(phen)_3]^{3+}$ ions from the DNA layer treating the sensor in solution of a high ionic strength (100 mM phosphate buffer pH 7.0) under stirring during 120 s. A negligible marker peak current was checked by the DPV record in blank.

DNA damage and effect of antioxidants were detected by the measurement of the DPV marker signal (as described above) after the pretreatment of the sensor in blank solution (peak current I_0 was obtained in triplicate) or in the cleavage mixture (2×10^{-4} M $FeSO_4$, 4×10^{-4} M EDTA, 9×10^{-3} M H_2O_2, in 10 mM phosphate buffer solution pH 7.0 without or with an antioxidant, application of the electrode potential of –0.5 V for 5 min.), (peak current I was obtained in triplicate) using one and the same DNA/SPE in order to compensate differences between individual DNA-modified strips.

The detection scheme used was as follows: three cycles of the I_0 current measurement /sensor regeneration – DNA damage (in the absence or presence of an antioxidant) - three cycles of the I current measurement/sensor regeneration. As the first measurement yielded usually somewhat lower peak current, the average signals I_0 and I were calculated from the second and third measurements and the normalized (relative) signal I/I_0 was obtained.

3. Results and discussion

3.1 Detection of DNA damage

The DNA redox marker $[Co(phen)_3]^{3+}$ can be accumulated effectively from its solution within the DNA layer on the electrode surface at both an open circuit [19-21, 24, 25] as well as a polarization of the electrode by a positive potential which is often used to stimulate association interactions of DNA [18, 26]. Voltammetric behavior of $[Co(phen)_3]^{3+}$ at the DNA-modified gold electrode [24], glassy carbon electrode [20], carbon paste electrode [25, 26] as well as carbon-based screen-printed electrode [18, 19] was reported previously. After the accumulation of the marker at DNA/SPE under the open circuit conditions, a good developed DPV peak of the complex appears at –0.130 V vs Ag/AgCl (Fig. 1). Depending on an ionic strength of the medium, intercalation (predominantly at high ionic strength) and electrostatic forces (predominantly at low ionic strength) take part in binding of the marker particles [20, 24]. The intercalation as well as electrostatic binding are equilibrium processes which can be utilized for a removal of $[Co(phen)_3]^{3+}$ from the DNA layer in solution without $[Co(phen)_3]^{3+}$ [19-21].

The marker signal obtained with the bare SPE after 120 s in the solution of $[Co(phen)_3]^{3+}$ represents about 20 % of the signal measured after the complex accumulation at the DNA-modified SPE (Fig. 1). Similarly to [18, 19], it indicates a contribution of the $[Co(phen)_3]^{3+}$ diffusion and adsorption at the bare SPE to the total DPV signal at the DNA-modified electrode. To evaluate only signal of the redox marker accumulated at DNA (peak current I_0), the total DPV peak current of $[Co(phen)_3]^{3+}$ obtained at

the DNA/SPE was corrected to its signal at the bare SPE by subtraction of the mean value of the last one.

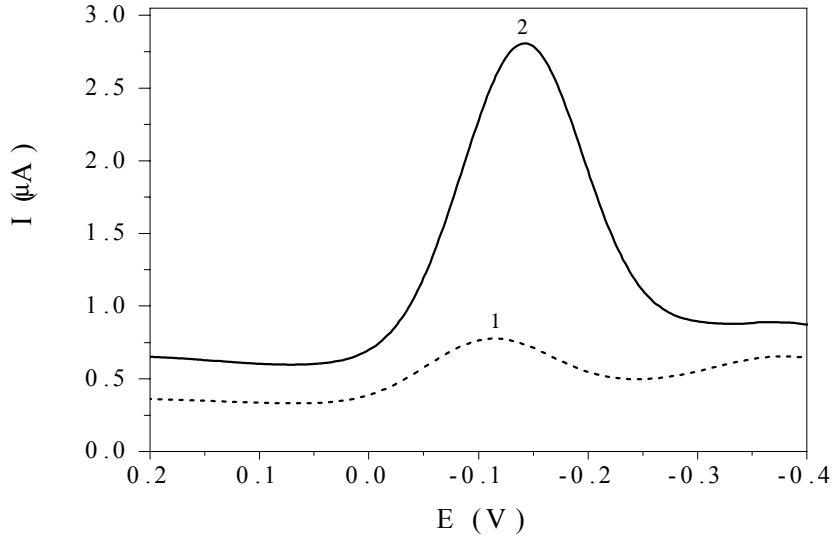

Figure 1. DPV curves of the $[Co(phen)_3]^{3+}$ complex in its 5×10^{-7} M solution obtained with SPE (1) and DNA/SPE (2) after 120 s accumulation at an open circuit, 10 mM phosphate buffer pH 7.0, pulse amplitude 100 mV, scan rate 25 mV/s.

The biosensor was immersed into 10 mM phosphate buffer containing $2x10^{-4}$ M $[Fe(EDTA)]^-$ ions and 9 mM H_2O_2 at aerobic conditions without bubbling air. With respect to the dominant presence of monoprotonated form of EDTA at pH 7.0 ($pK_{a,2}$ = 6.7), its iron(II) complex is denoted here as $[Fe(EDTA)]^-$. A negative polarization potential was switched on for 5 min to control the reduction/oxidation cycle of the iron atom. The signal obtained with DNA/SPE after such preliminary treatment of the sensor in the cleavage mixture followed by the transfer of the electrode to the $[Co(phen)_3]^{3+}$ solution and the accumulation of the marker decreases from the peak current I_0 to I.

Utilizing an oxidation signal of the DNA guanine moiety it was shown previously that this decrease in the marker signal is due to a deep change in the dsDNA structure which involves DNA strand breaks and leads to a decrease of its ability to bind the $[Co(phen)_3]^{3+}$ [16-18]. To compensate differences in the properties of individual strips of the DNA-modified screen-printed electrodes, one and the same DNA/SPE was used in our measurements for the evaluation of a couple of the I_0 and I peak currents corresponding to an individual composition of the cleavage mixture and the marker signal was expressed by its normalized (relative) value I/I_0.

This I/I_0 signal has reached the values of 0.79 and 0.02 for the potential of –0.3 and –0.5 V, respectively, applied to the biosensor during its treatment in the cleavage mixture. In blank solution containing oxygen and with an electrode potential of –0.5 V, the relative marker signal was 0.71, while with 9 mM H_2O_2 and the same electrode potential the signal decreased to 0.15. Thus, the application of the sufficiently negative electrode potential of –0.5 V can be utilized advantageously for the reduction of iron and simplification of the chemical composition of the cleavage mixture omitting chemical

reductant. This is of interest particularly at the evaluation of mixed samples of the natural antioxidants containing also ascorbic acid.

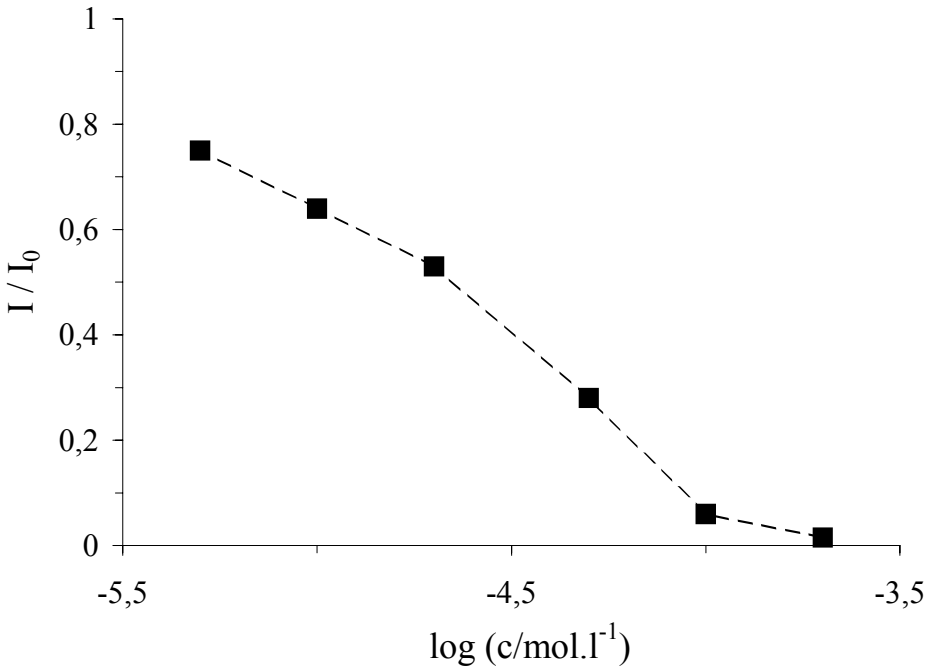

Figure 2. Dependence of the marker relative DPV signal at the DNA/SPE on the concentration of $FeSO_4$ in the cleavage mixture: $FeSO_4$, 4×10^{-4} M EDTA, 9×10^{-3} M H_2O_2 in 10 mM phosphate buffer pH 7.0, the electrode potential of –0.5 V, incubation for 5 min. Other conditions as in Figure 1.

The cleavage activity of the reaction medium against the DNA/SPE biosensor depends on the concentration of the iron ions. The calibration graph for the $[Fe(EDTA)]^-$ complex ions is depicted on Fig. 2. The strip-to-strip repeatability for the determination of 2×10^{-4} M $[Fe(EDTA)]^-$ at single-use DNA/SPE sensors was characterized by the relative standard deviation of 19 % (n=8). With respect to the high efficiency of 2×10^{-4} M iron complex towards DNA damage, this concentration and the incubation time of 5 min were chosen for the evaluation of antioxidants.

3.2 Detection of antioxidative activity of standard materials and plant extracts

The rosmarinic and caffeic acids were found as the main constituents of phenolic acids in the plant extracts under study using the HPLC technique and the procedure reported previously [22]. The concentrations of these acids are presented in Table 1.

The antioxidative activities of rosmarinic and caffeic acids as the standards as well as that of four plant extracts were examined by addition to the cleavage mixture with 10 % methanol (Figs. 3 and 4). It was tested in a blank experiment that this methanol content has no effect on the DNA/SPE sensor and, consequently, the redox marker signal.

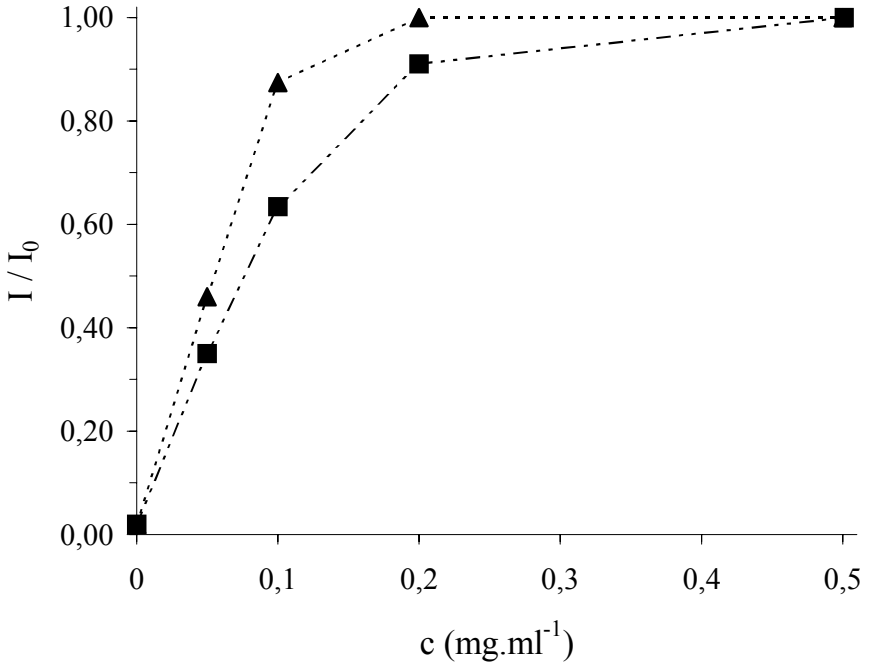

Figure 3. Antioxidative effect of rosmarinic acid (▲) and caffeic acid (■) in cleavage mixture on the relative marker signal at the DNA/SPE. Incubation of the sensor in $2x10^{-4}$ M $FeSO_4$, $4x10^{-4}$ M EDTA, $9x10^{-3}$ M H_2O_2 in 10 mM phosphate buffer pH 7.0 with10 % of methanol at the electrode potential of –0.5 V for 5 min. Other conditions as in Figure 1.

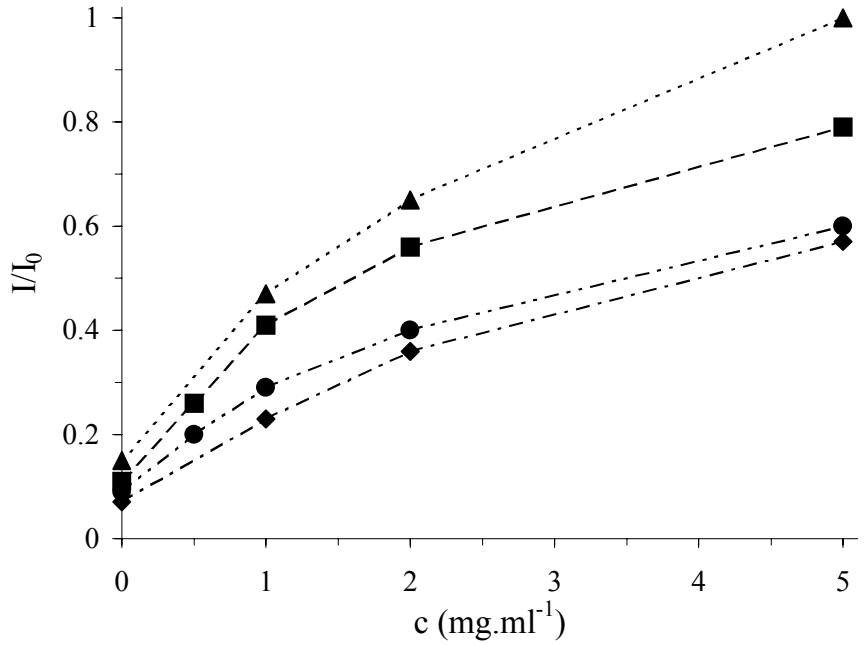

Figure 4. Antioxidative effect of plant extracts in cleavage mixture on the relative marker signal at the DNA/SPE: lemon balm (▲), oregano (■), thyme (●) and agrimony (◆). Other conditions as in Figure 3.

Table 1. HPLC determination of the phenolic acids in the aqueous plant extracts (2g dry plant /100ml).

Plant material	**Concentration of acid (mg/g of the dry plant)**	
	Rosmarinic acid	Caffeic acid
Lemon balm	4.24	1.50
Oregano	25.70	2.12
Thyme	not detectable	1.19
Agrimony	0.16	not detectable

In Figs. 3 and 4, a strong protection of surface attached DNA from its damage is clearly shown which confirms the antioxidative activity of all tested samples. The effect of antioxidants increases with their concentration (given as mg of the dry plant added in the form of the extract per 1 ml of the cleavage mixture) and the curves level off at high concentrations. According to the saturation level, the activity of the plant extracts under study can be ordered as follows: lemon balm > oregano > thyme > agrimony. The antioxidative activity of the plant extracts for a given concentration of dry plant in the cleavage mixture, for instance, 1.0 mg/ml, was also expressed in the terms of the concentration of the standard (rosmarinic acid) which leads to the same I/I_0 value. The results obtained with the DNA biosensor are shown in Table 2.

Table 2. Antioxidative activity of the plant extracts with the concentration of 1.0 mg dry plant / ml in the cleavage mixture expressed in the terms of the concentration of rosmarinic acid in the cleavage mixture leading to the same value of the I/I_0 relative signal of the DNA marker (obtained using Figs. 3 and 4).

	Lemon balm	**Oregano**	**Thyme**	**Agrimony**
Equivalent of rosmarinic acid (mg/ml)	0.046	0.039	0.028	0.021

Table 3. Antiradical activity of the plant extracts determined spectrophotometrically with the DPPH radical. The efficient concentration (EC_{50}, in g antioxidant/g DPPH) and the antiradical power (ARP = $1/EC_{50}$).

	Rosmarinic acid	**Caffeic acid**	**Lemon balm**	**Oregano**	**Thyme**	**Agrimony**
EC_{50}	0.10	0.09	1.70	1.12	4.42	2.49
ARP	10.4	11.1	0.59	0.90	0.23	0.40

To validate the assay, antiradical measurements have been performed using the spectrophotometric method with the DPPH radical as reported previously [6, 7]. The antiradical activity of the plant extracts was expressed using conventional parameters such as the efficient concentration necessary to decrease the initial DPPH concentration by 50 % (EC_{50}) and the antiradical power (ARP equal to $1/EC_{50}$). The larger is ARP, the more efficient the antioxidant. The EC_{50} and ARP values obtained are summarized in Table 3. Taking into the account small differences in the antioxidative activity of both

standard acids as well as some couples of the plant extracts (lemon balm - oregano and thyme - agrimony), the antioxidative activity determined with the electrochemical sensor follows generally the order of the antiradical activity found with DPPH. The orders of antiradical activity and antioxidative activity of the plant extracts follow quite good the concentration of the phenolic acids which can be taken as an evidence for their contribution to the above effects.

It is clear from the literature that the antioxidative activity is operationally defined. For instance, changes in order of the antioxidant activity of caffeic acid, rosmarinic acid and related compounds according to experimental conditions used at the lipid oxidation in the Rancimat method were reported [27]. Moreover, a higher activity of rosmarinic acid than that of caffeic acid was also found using the DPPH method [27].

Conclusion

The behavior of the DNA/SPE sensor was investigated with respect to the $[Fe(EDTA)]^-$ based chemical nuclease under conditions of the electrochemical reduction of the central metal atom and generation of reactive oxygen species. The standards of rosmarinic and caffeic acids as well as plant extracts containing these phenolic acids, when present in the cleavage mixture, protect effectively DNA from its degradation. The DNA biosensor represents a suitable tool for the simple detection of antioxidants and for the evaluation of the antioxidative activity of the plant extracts used in food technology.

Thus, electrochemical measurements can provide valuable additional information on the presence of phenolic antioxidants to the investigation by the conventional spectrophotometric method with the DPPH radical and the disposable low-cost electrochemical biosensor based on the DNA-modified screen-printed electrode can be applied as simple test device to routine analysis.

Tests of the DNA/SPE sensors for the evaluation of other groups of natural antioxidants such as flavonoids, exhibiting also prooxidant effects under certain conditions, are in progress.

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Sample Availability: Available from the author.